Extent and character of circadian gene expression in *Drosophila melanogaster*: identification of twenty oscillating mRNAs in the fly head

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Background: Although mRNAs expressed with a circadian rhythm have been isolated from many species, the extent and character of circadianly regulated gene expression is unknown for any animal. In *Drosophila melanogaster*, only the *period (per)* gene, an essential component of the circadian pacemaker, is known to show rhythmic mRNA expression. Recent work suggests that the encoded Per protein controls its own transcription by an autoregulatory feedback loop. Per might also control the rhythmic expression of other genes to generate circadian behavior and physiology. The goals of this work were to evaluate the extent and character of circadian control of gene expression in *Drosophila*, and to identify genes dependent on *per* for circadian expression.

Results: A large collection of anonymous, independent cDNA clones was used to screen for transcripts that are rhythmically expressed in the fly head. 20 of the 261 clones tested detected mRNAs with a greater than two-fold daily change in abundance. Three mRNAs were maximally expressed in the morning, whereas 17 mRNAs were most abundant in the evening — when *per* mRNA is also maximally expressed (but when the flies are inactive). Further analysis of the three 'morning' cDNAs showed that each has a unique dependence on the presence of a light-dark cycle, on timed feeding, and on the function of the *per* gene for its oscillation. These dependencies were different from those determined for *per* and for a novel 'evening' gene. Sequence analysis indicated that all but one of the 20 cDNAs identified previously uncloned genes.

Conclusions: Diurnal control of gene expression is a significant but limited phenomenon in the fly head, which involves many uncharacterized genes. Diurnal control is mediated by multiple endogenous and exogenous mechanisms, even at the level of individual genes. A subset of circadianly expressed genes are predominantly or exclusively dependent on *per* for their rhythmic expression. The *per* gene can therefore influence the expression of genes other than itself, but for many rhythmically expressed genes, *per* functions in conjunction with external inputs to control their daily expression patterns.

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Background

Much of the behavior and physiology of the *Drosophilidae* fruit flies is temporally organized around a 24-hour day. Eclosion from the pupal case, for example, occurs exclusively in a brief window of time in the early morning [1]. Locomotor activity also has a strong diurnal rhythm, with substantially greater levels of activity occurring during the day than at night [2]. These circadian rhythms are endogenously generated, and persist in the absence of all external timing cues (see [3] for review).

The temporal organization of physiology and behavior has a discrete genetic basis. Specific genes appear to function primarily in organizing behavior around an approximately 24-hour clock. Mutations in the *period (per)* [4] and *timeless* [5,6] genes can completely disrupt the daily organization of adult locomotion and pupal eclosion without disrupting the actual behaviors themselves. Animals mutant in these genes are fully viable and without any obvious morphological defects.

Recent studies have begun to elucidate how single genes can so profoundly affect the temporal organization of behavior. The *per* gene encodes a predominantly nuclear protein (Per) that is expressed in the fly brain [7], with homology in a dimerization domain (the PAS domain) to several transcription factors [8]. Both *per* mRNA and protein oscillate with a free-running circadian rhythm [9–11]. The *per* mRNA reaches peak levels in the evening, and Per protein reaches its peak 8–10 hours later [12,13]. The oscillation of *per* mRNA depends on the intact function of Per protein [11] — a feedback loop which has been suggested to comprise the essential core of the circadian pacemaker [11,14–16]. Heat-shock induction of *per* mRNA in a wild-type background shifts
the phase of the circadian clock, with the phase of the resultant rhythm being dependent on the phase of heat-shock induction [17]. This result strongly suggests that the instantaneous level of per mRNA in part determines the phase of the circadian clock. Thus, the circadian expression of the per gene is apparently essential to the function of the endogenous circadian pacemaker.

Although per is the only gene in Drosophila melanogaster (D. melanogaster) that is known to undergo circadian rhythms of expression, genes expressed with diurnal and circadian rhythms have been identified in many other organisms from cyanobacteria to mice (reviewed in [18]). These genes function in many physiological processes, including light transduction [19,20], endocrine function [21-23] and metabolism [24]. Most of these oscillating genes were identified by the testing of various known genes for rhythmic expression, or by the fortuitous observation of diurnal oscillation of a known gene product.

The extent and importance of circadian control of gene expression are not well known. The only previous large-scale survey was reported recently for a prokaryotic cyanobacterium, Synechococcus [25]. By monitoring luciferase expression in individual Synechococcus colonies carrying random insertions of a luciferase reporter gene, the authors found that almost all of the 800 colonies analyzed showed rhythmic expression. The only other systematic search for circadianly expressed genes used a two-timepoint subtractive hybridization approach in the mold Neurospora crassa; from a pool of total Neurospora mRNA, the authors found two cDNAs undergoing circadian rhythms of expression [26]. Although this study did not identify all genes under circadian control in Neurospora (see [27]), the results nevertheless suggest that circadian control of gene expression is a quite limited phenomenon in Neurospora. No comparable study has been reported for Drosophila or any other animal.

The present study was initiated to answer two questions. First, what is the extent of diurnal control of gene expression in D. melanogaster? Second, to what extent is diurnal gene expression dependent on the function of the per gene, and to what extent is it dependent on external time cues? To begin to answer these questions, we undertook a screen through a large collection of independent cDNAs known to be expressed in the adult Drosophila head but not in the early embryo, searching for mRNAs that demonstrate diurnal variations in abundance. In addition to answering the questions of extent and mechanism of circadian control of gene expression, we also hoped to identify additional circadianly controlled genes that might contribute to clock function and to its control of physiology and behavior. Here, we describe the results of the screen, and report on further circadian and molecular characterization of the subset of the genes that were expressed at their highest levels in the morning.

Results

A screen for diurnally expressed mRNAs in the fly head

By performing a subtractive hybridization between cDNA libraries prepared from adult Drosophila heads and 0–1-hour-old embryos, Palazzolo et al. [28] isolated and characterized 436 independent cDNAs that were expressed in the adult fly head but not in the early embryo. This collection of anonymous cDNA clones is well suited for screening for diurnally expressed genes. The collection contains cDNAs that correspond to a wide range of transcript abundances, from transcripts expressed at nearly 1% of fly head mRNA to those expressed at only several parts per million of fly head mRNA. Because the 0–1 hour embryo does not have a nervous system, the collection is enriched for genes specific to the nervous system, and the representation of ubiquitously expressed genes is reduced. Importantly, the procedure used in characterizing these transcripts eliminated redundancy in the collection, as cross-hybridizing clones were identified and removed. Of the 436 cDNAs in the collection, 280 show ‘simple’ expression patterns manifest on Northern blots by one or more transcripts that are coordinately regulated throughout development. The remaining cDNAs identify multiple transcripts that are not coordinately regulated during development.

To determine the diurnal expression patterns of these genes, adult D. melanogaster Canton-S (wild-type) flies were collected from large population cages at Zeitgeber times (ZTs) 2, 8, 14 and 20, in a 12-hour light–12-hour dark (LD 12:12) cycle (by convention, ZT 0 occurs at the dark–light transition). Total RNA was prepared from the heads of these flies. A simple, rapid procedure was developed for the synthesis of single-stranded, radio-labeled RNA probes from polymerase chain reaction (PCR) amplification products of each of the ‘simple’ expression pattern cDNA phage clones (see Materials and methods). Each of these cRNAs was used to probe individual northern blots of RNA from each of the four Zeitgeber times. A per probe was used in the experiments as a positive control for rhythmic mRNA expression, and probes for the rp49 mRNA, which encodes a ribosomal protein, and for the ninaE mRNA, which encodes an abundant opsin, served as non-oscillating negative controls.

The vast majority (> 90%) of the 261 cDNAs detected mRNAs that were expressed constitutively or that cycled very weakly (such as the 7C12 cDNA probe; Fig. 1). 20 cDNA probes, however, identified oscillating mRNAs with greater than two-fold peak-to-trough differences in expression over the 24-hour period (Fig. 1). Each of the probes that detected cycling mRNAs was retested on northern blots of RNA from a separate population of flies to confirm the oscillation of expression. We named this collection of cycling mRNAs the Dregs, for Drosophila rhythmically expressed genes, and assigned each a number according to its overall level of expression in the adult fly head (Dreg-1 was the most abundant).
The 20 diurnally oscillating mRNAs display two general temporal expression patterns

The Dregs displayed two general temporal patterns of expression. All showed a single daily peak and a single trough of expression. Dreg-1, Dreg-2 and Dreg-3 showed highest levels of expression in the early morning or late at night just before day break (Fig. 1); these are referred to as the ‘morning’ Dregs hereafter. Dreg-1 and Dreg-3 were expressed with peak levels at ZT 2 and troughs at ZT 14, in the opposite phase from per mRNA. Dreg-2 followed a similar temporal expression pattern, but demonstrated minimal transcript levels at ZT 20, rather than ZT 14. The other 17 Dregs all showed highest expression in the early night, at ZT 14, and lowest expression at ZT 2, in the same phase as expression of per mRNA. These are referred to as the ‘evening’ Dregs hereafter. Several Dregs showed multiple poly-adenylated transcripts on northern blots (Fig. 1; Table 1). In some instances (such as Dreg-10 and Dreg-16), the multiple transcripts all showed diurnal rhythmicity. In other instances (such as Dreg-9, Dreg-12 and Dreg-21), one transcript cycled while the other was constitutively expressed.

The amplitude of oscillation of the Dregs was comparable to, and in some cases greater than, the five-fold peak-to-trough amplitude that was observed for per mRNA. Dreg-2 showed the lowest amplitude of oscillation, of about 2.5-fold, while several of the Dregs that were expressed in phase with per mRNA (such as Dreg-9) showed amplitudes as high as 50-fold. These values are not absolutes, however, as the amplitude of mRNA oscillation for many of the Dregs, as well as for per, is influenced by environmental conditions (see below).

Both Dreg-1 and Dreg-2 were expressed in the body of the fly as well as in the head, whereas Dreg-3 mRNA was found exclusively in the head (Table 1). Among the evening Dregs, only Dreg-6 and Dreg-9 transcripts were

![Fig. 1. Diurnal expression of the Dreg mRNAs in fly heads. Each panel shows an individual northern blot of total head RNA (20 μg per lane) from flies kept under LD 12:12 lighting conditions and sacrificed at (lane 1) ZT 2, (lane 2) ZT 8, (lane 3) ZT 14 or (lane 4) ZT 20. The lane on the far right of each panel is poly-A+ mRNA (≈1 μg) prepared from all time points. Blots were hybridized with probes from Dreg-1 to Dreg-21, cDNA clone 7C12, ninaE, rp49 and per, as indicated. Blots were exposed for different times to obtain the autoradiograms shown; the estimated abundance (based on exposure time and probe lengths) and sizes of the poly-A+ transcripts are given for each Dreg in Table 1. 7C12 is an example of one of the many non-cycling genes identified in the screen; the sizes of its transcripts are 2.2 kb and 6.2 kb. The per, ninaE and rp49 genes were used throughout the screen as cycling (per) and non-cycling (ninaE, rp49) control mRNAs. Dreg-1 to Dreg-3 show highest expression in pre-dawn or early morning; Dreg-5 to Dreg-21 show highest expression in the evening.](image)
detected (albeit weakly) in polyA+ RNA from fly bodies. All of the Dregs were expressed in the heads of eyes absent (eya) mutant flies [29], indicating that none of these genes are expressed exclusively in the compound eye.

There was an interesting and unexpected correlation between the absolute levels of expression of the Dregs and their temporal expression patterns. The three morning genes were of relatively high abundance (~0.05–0.1% head mRNA; Table 1). In contrast, the evening Dregs were universally of low abundance — approximately 100–1000 times less abundant than the morning Dregs. The majority of evening mRNAs were also found to be large transcripts, with all except Dreg-5 and Dreg-15 being 6 kb or greater in length.

Oscillations of the morning Dregs show distinct, complex dependencies on light, feeding time and the per gene

Because the original screen for cycling genes used flies maintained in a light-dark cycle, we expected to identify mRNAs that are responsive to light as well as those that fluctuate with an endogenous circadian rhythm, independent of external time cues (Zeitgebers). The flies that were used in the initial screening experiments were subject to three identifiable sources for their daily rhythmicity: the lighting cycle, the daily feeding time and the endogenous circadian pacemaker. We tested the three morning Dregs for their dependence on each of these potential influences. Surprisingly, the dependencies of each of the morning Dregs were different and complex; these dependencies were also different from the dependencies of per and of one of the evening genes (see Discussion). The results are summarized in Table 2 and are described in detail below.

**Dreg-1 and Dreg-3 mRNAs continue to oscillate in the absence of a light-dark cycle**

We first tested whether Dreg-1, Dreg-2, Dreg-3 and per mRNAs would oscillate in abundance in the absence of a light-dark cycle. Dreg-1, Dreg-3 and per mRNAs continued to oscillate with constant period and slightly reduced amplitude in the transition from light–dark cycle to total darkness (Fig. 2); these genes therefore do not have an absolute requirement for light to drive their circadian cycling. Dreg-6 to Dreg-10 and Dreg-15 also continued to cycle in the transition from the light–dark cycle to total darkness (data not shown). (We have not, however, eliminated the possibility that the cycling observed in these experiments is due to ‘after effects’ of the lighting cycle [30]). In contrast, the diurnal oscillation of Dreg-2 mRNA was dependent on the light–dark cycle, in an

### Table 1. Size, abundance and distribution of Dreg mRNAs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>cDNA size (bp)*</th>
<th>Size of poly-A+ mRNAs (kb)</th>
<th>Estimated fraction of head mRNA (%)†</th>
<th>Expressed in body‡</th>
<th>Expressed in 24 h embryo§</th>
<th>Sequence¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dreg-1</td>
<td>350</td>
<td>1.0</td>
<td>0.2</td>
<td>Yes</td>
<td>No</td>
<td>Adh</td>
</tr>
<tr>
<td>Dreg-2</td>
<td>465</td>
<td>1.0</td>
<td>0.1</td>
<td>Yes</td>
<td>No</td>
<td>Novel</td>
</tr>
<tr>
<td>Dreg-3</td>
<td>310</td>
<td>2.7</td>
<td>0.05</td>
<td>No</td>
<td>Yes</td>
<td>Novel</td>
</tr>
<tr>
<td>Dreg-5</td>
<td>385</td>
<td>1.8</td>
<td>0.001</td>
<td>No</td>
<td>Yes</td>
<td>Novel</td>
</tr>
<tr>
<td>Dreg-6</td>
<td>310</td>
<td>7.0</td>
<td>0.0005</td>
<td>Weakly</td>
<td>No</td>
<td>New</td>
</tr>
<tr>
<td>Dreg-7</td>
<td>360</td>
<td>8.2</td>
<td>0.0005</td>
<td>No</td>
<td>Yes</td>
<td>New</td>
</tr>
<tr>
<td>Dreg-8</td>
<td>540</td>
<td>7.4</td>
<td>0.0005</td>
<td>No</td>
<td>Yes</td>
<td>New</td>
</tr>
<tr>
<td>Dreg-9</td>
<td>800</td>
<td>7.0</td>
<td>0.0005</td>
<td>Weakly</td>
<td>No</td>
<td>New</td>
</tr>
<tr>
<td>Dreg-10</td>
<td>460</td>
<td>6.9</td>
<td>0.0001</td>
<td>No</td>
<td>No</td>
<td>New</td>
</tr>
<tr>
<td>Dreg-11</td>
<td>480</td>
<td>6.6</td>
<td>0.0005</td>
<td>No</td>
<td>Yes</td>
<td>New</td>
</tr>
<tr>
<td></td>
<td></td>
<td>smear</td>
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<td></td>
<td></td>
<td></td>
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<td>Dreg-12</td>
<td>290</td>
<td>9.0</td>
<td>0.00005</td>
<td>No</td>
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<td>New</td>
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<tr>
<td>Dreg-13</td>
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<td>0.00005</td>
<td>No</td>
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<td>New</td>
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<tr>
<td>Dreg-14</td>
<td>375</td>
<td>6.2</td>
<td>0.00005</td>
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<tr>
<td>Dreg-15</td>
<td>650</td>
<td>3.5</td>
<td>0.00005</td>
<td>No</td>
<td>Yes</td>
<td>New</td>
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<tr>
<td>Dreg-16</td>
<td>350</td>
<td>7.3</td>
<td>0.00005</td>
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<td>Yes</td>
<td>New</td>
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<tr>
<td>Dreg-17</td>
<td>900</td>
<td>10.0</td>
<td>0.00005</td>
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<td>Yes</td>
<td>New</td>
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<tr>
<td>Dreg-18</td>
<td>100</td>
<td>14.0</td>
<td>0.00005</td>
<td>No</td>
<td>Yes</td>
<td>New</td>
</tr>
<tr>
<td></td>
<td></td>
<td>smear</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dreg-19</td>
<td>960</td>
<td>10.0</td>
<td>0.00005</td>
<td>No</td>
<td>Yes</td>
<td>New</td>
</tr>
<tr>
<td>Dreg-20</td>
<td>560</td>
<td>6.0</td>
<td>0.00005</td>
<td>No</td>
<td>Yes</td>
<td>New</td>
</tr>
<tr>
<td>Dreg-21</td>
<td>340</td>
<td>6.9</td>
<td>0.00005</td>
<td>No</td>
<td>No</td>
<td>New</td>
</tr>
</tbody>
</table>

*Size of XbaI–EcoRI cDNA insert in the original clones from the lambda-SWAI cDNA library. † Abundance was estimated from exposure times required to obtain a signal approximately equal to ninaE, taking into account differing probe lengths. ninaE represents ~1% of fly head mRNA [47]. ‡ Body RNA assessments were made on 2 μg poly-A+ body mRNA prepared from the same flies as used for the fly head Northern blots, pooled for all time points. § See [28]. ¶ DNA sequence (>200 bp) was obtained from each cDNA insert and will be deposited in GeneBank. ‘New’ indicates no matches were found in GeneBank for the partial cDNA. Novel indicates no closely related proteins were found for the coding sequence of a complete cDNA.
interesting manner. This mRNA displayed increased expression from ZT 20–26 in the first cycle of total darkness. However, the transcript levels of Dreg-2 remained high and without fluctuation throughout the remainder of the cycle. This suggests that morning light is required to effect the evening decline in Dreg-2 transcript levels under these conditions, and that Dreg-2 expression is not driven directly by the endogenous circadian pacemaker.

The phase of morning Dreg expression is altered by the time of feeding

Flies kept in large cages require regular replenishment of food. In our screen, a fresh food tray was added each day at ZT 17, during the subjective night (although some food remained available to the flies at all times). To determine whether the addition of fresh food acted as a Zeitgeber for the expression of the Dregs, we added fresh food trays at ZT 1 instead of ZT 17. The peak expression of all three morning Dregs shifted to later in the day (ZT 8), and the trough of expression shifted to ZT 2 (Fig. 3). In contrast, this change had no effect on the phase or amplitude of per mRNA cycling (Fig. 3) or on the phase of expression of Dreg-5 to Dreg-9 ([31] and data not shown). These data demonstrate that the timing of the placement of the food trays is sufficient to alter the phase of expression of all three morning Dregs, and to dissociate their relative phase from that of the per mRNA and several other cycling genes.

To determine whether the timed introduction of fresh food was required for the cyclic expression of the morning Dregs, we raised flies in small bottles with abundant food and allowed them to feed ad libitum. Dreg-1, Dreg-3 and per continued to demonstrate diurnal cycling in these conditions (Fig. 4a,c,d), but the amplitude of oscillation was reduced by about half. Thus, although the timing of food availability can alter the phase of Dreg-1 and Dreg-3 rhythmicity, it is not necessary for manifest rhythmicity. Dreg-2 did not show significant oscillations in flies housed in small bottles (Fig. 4b), and therefore requires both a light–dark cycle and timed food availability for its rhythmicity in wild-type flies.

In a light–dark cycle, the function of the per gene is required for the oscillation of Dreg-1 but not Dreg-3

One of the goals of our screen was to identify genes that are controlled by the per locus. We therefore tested whether the morning Dregs would oscillate in per null mutants. The per allele used here, per01, is a point mutation that eliminates per function and produces flies with no apparent circadian rhythms [32]. The temporal expression patterns of the morning Dregs and per in wild-type Canton-S and mutant y per01 strains in LD 12:12 are shown in Figure 4. The per mRNA was expressed in the mutants but remained at a constant level throughout the circadian cycle (Fig. 4d), confirming the results of Hardin et al. [11]. Similarly, the oscillation of Dreg-1 mRNA ceased in the per01 flies, indicating that circadian oscillation of Dreg-1 also requires wild-type per function (Fig. 4a). In contrast, Dreg-3 oscillated in a similar manner in the presence or absence of per function (Fig. 4c). This transcript thus has no dependence on the per gene for its oscillation under these conditions.
Fig. 3. Effect of altered feeding time on the cycling mRNA expression of the morning Dregs and per. Northern blots of total head RNA from flies maintained in LD 12:12 as in Figure 1, except that for half of the cages food trays were swapped at ZT 1 (right panels) instead of the standard time of ZT 17 (left panels). Arrowheads indicate the time of food-tray swapping. Quantification of the expression of each gene (as described in the legend to Fig. 4) showed that signal amplitudes did not differ significantly under the two feeding conditions.

The results with Dreg-2 in this experiment were more complex. In wild-type flies maintained in small bottles and in a LD 12:12 lighting cycle, this gene did not show significant oscillation (Fig. 4b). Surprisingly, Dreg-2 did show weak rhythmicity in per0 flies kept in the same conditions, with increased expression in the early day (Fig. 4b). Taken together with the previous results for Dreg-2, this indicates that the diurnal control of Dreg-2 expression is a complex function of the interplay between light–dark cycle, per gene function and feeding conditions.

In addition to its effects on the oscillatory behavior of the Dregs, per also affected the overall level of expression of some of the Dregs. Significantly more Dreg-1 transcript was found in wild-type flies than in per0 flies at all times of the day, and particularly at those times when Dreg-1 was maximally expressed (Fig. 4a). Hence, per appears to potentiate the expression of Dreg-1. In contrast, wild-type per suppressed Dreg-3 mRNA expression at all times of the day [31], and it potentiated and suppressed per mRNA expression at different times of the day (Fig. 4d). No significant effects of per genotype on the overall levels of expression of Dreg-2 or Dreg-3 were detected. Thus, per affects the expression of several cycling genes, but it appears to do so by modulating their patterns of expression in different ways. This conclusion should be considered provisional, however, as the per0 and wild-type flies used in this experiment were not fully isogenic and we cannot exclude the possibility that other genetic differences between the strains may have contributed to differences in expression levels.

To determine whether the observed oscillation of Dreg-2 and Dreg-3 expression in per0 flies was driven by the LD 12:12 lighting conditions or reflected the presence of a per-independent endogenous oscillator, we tested the circadian cycling of these cDNAs in per0 flies kept in constant darkness (DD). Neither Dreg-2 nor Dreg-3 expression oscillated under these conditions (data not shown). The cycling of these genes in per0 mutants is therefore attributable to an effect of light on their expression that is independent of the effect of the per gene.

**Sequence analysis of the Dreg genes**

As an initial step towards understanding the functions of the Dreg genes, we obtained partial sequences of the entire set of Dregs and complete coding sequences for the three morning Dregs. All of the original Dreg cDNA clones were relatively short (~200–1000 bp; see Table 1) and probably represent the 3' ends of their respective transcripts, as each is terminated by a poly-A sequence. We sequenced the ends of each Dreg cDNA clone to establish identities with previously cloned *Drosophila* genes. Only one of the 20 Dreg genes corresponded to a known gene: the Dreg-1 sequence precisely matched the 3' portion of the *Drosophila* alcohol dehydrogenase (Adh) gene, establishing that Dreg-1 is Adh (Table 1). The remaining 19 Dregs did not show sequence identity with any cDNA or genomic sequences in the GeneBank or EMBL databases, indicating that all of these Dregs represent newly identified *Drosophila* genes (Table 1).

To obtain the complete coding sequence of the two other morning genes, full-length Dreg-2 and Dreg-3 cDNA clones were isolated from adult *Drosophila* cDNA libraries and the sequences of the inserts were determined. The sequence of Dreg-2 is shown in Figure 5a. The Dreg-2 cDNA encodes a predicted 260-residue protein of novel sequence. Searches of GeneBank and EMBL databases with the BLAST, Blaze and FastDB programs revealed no significant homologies, except for a weak homology to a conserved region of phenylalanine-ammonia lyase.
Effects of a per null mutation on the cycling expression of the morning Dregs. Canton-S flies and y per^0 flies were entrained for 4 days under LD 12:12 conditions in small bottles on cornmeal-molasses agar covered with yeast. In the next cycle, head RNA was prepared from the wild-type and mutant flies sacrificed at the indicated circadian times. Individual northern blots of the RNA were probed for (a) Dreg-1, (b) Dreg-2, (c) Dreg-3 and (d) per, and the level of each mRNA was quantified on a phosphorimager. Values shown are the mean and standard error of six independent experiments. Each point was normalized first to rp49 expression (to control for any differences in RNA loading) and then to the maximum expression for that gene using the method of [11]. The same data are plotted twice over two cycles to facilitate comparison of waveforms.

**Discussion**

In this study, we have screened a large, heterogeneous collection of anonymous cDNA clones for those that show daily rhythmic expression. We identified 20 genes that showed significant cycling, only one of which (Dreg-1) corresponds to a gene that has been cloned previously. We further characterized the three genes (Dreg-1, Dreg-2 and Dreg-3) that are maximally expressed at predawn or early morning. Elsewhere, we examine in detail one of the 17 evening genes identified in the screen, Dreg-5, which is expressed strictly in phase with per mRNA [31]. Comparison of the oscillatory behavior of these four genes, as well as per, demonstrates a surprising variety of diurnal regulatory controls: each of these five cycling genes shows a unique dependence on the endogenous circadian oscillator and on external time cues, ranging from complete dependence on the per gene to predominant dependence on external time cues.

**The extent of oscillating gene expression in Drosophila**

Our screening experiment allows us to estimate the prevalence of oscillating transcripts in the fly head. The results indicate that a small but significant proportion of transcripts specific to the adult fly undergo diurnal rhythms of expression: 8% of the genes in the collection demonstrated greater than two-fold oscillations of abundance. This estimate is based on flies housed in large population cages and kept in LD 12:12 lighting conditions, and it does not include genes that cycle in only a subset of the head tissues in which they are expressed — such cycling would not have been detected in our screen.

Our experiments further suggest that the number of cycling genes in Drosophila is not fixed but rather differs under different environmental conditions. Several genes that we identified altered their cycling behavior or ceased to cycle under conditions that were different from those used in the screen. For example, Dreg-2 mRNA levels cycled in flies kept in LD 12:12 in large population cages, but did not oscillate in flies housed in small bottles under the same lighting regimen. Furthermore, initial experiments with the evening Dregs (Dreg-5 to Dreg-21) have shown that cycling in at least a subset of these genes is also highly dependent on environmental conditions. Several evening Dregs showed robust cycling in large population cages, but oscillated with diminished amplitude or failed to cycle when the flies were kept in small bottles (R.N.V.G. and H.B., unpublished observations). It also seems likely that some of the genes that did not
show cycling behavior in the population cages might oscillate in flies kept in other environmental conditions. Thus, our estimate of the extent of circadian gene expression in Drosophila heads should not be interpreted as an absolute value but rather as a representative value for a standard set of environmental conditions.

Our estimate of the prevalence of rhythmically expressed genes in Drosophila heads differs dramatically from the result of the only other large-scale survey of circadian gene expression that has been conducted. Liu et al. [25] reported recently that nearly every random insert of a reporter construct in the cyanobacterium Synechococcus genome manifests significant circadian rhythmicity in expression. Our results show that the Drosophila genome is not subject to such global circadian control, and that the result from Synechococcus is unlikely to be general. Some of the difference between the estimates for the two organisms may be attributable to biases in the sets of genes surveyed, to the different assays of gene expression used, or to specific environmental conditions used in the experiments. But it is possible that much of the difference is real and reflects extreme differences in the circadian control of gene expression, behavior and physiology between the two organisms. It should be noted that our reporter construct in the cyanobacterium Synechococcus was used as a negative control, as the first methionine in the long open-reading frame of the mold Neurospora crassa.

The only other systematic search for circadianly regulated transcripts used a subtractive hybridization approach in the mold Neurospora crassa. Two cycling transcripts, called ccg-1 and ccg-2, were identified [26,36], indicating that circadian control of gene expression is a limited phenomenon in Neurospora, as in Drosophila. Another Neurospora gene, frequency (frq), which has many functional similarities and weak sequence similarity to per, has recently been shown to undergo autoregulated circadian oscillation in a manner analogous to that observed for per [27]. Unlike per, however, frq mRNA is maximally expressed during daytime. Both ccg-1 and ccg-2 also show maximum expression during daytime, and both are at least partially dependent on frq for their circadian rhythmicity [37]. Thus, in both the work of Loros et al. [36] on Neurospora and our study on Drosophila, the extent of circadian control of gene expression appears to be limited to a relatively small proportion of the genome, and the majority of identified cycling transcripts are expressed in the same phase as the transcripts of a gene known to be essential to the function of the circadian clock.

The diverse character of cycling expression in Drosophila

Diurnal control of gene expression in Drosophila is not a unitary phenomenon. Diurnal control of the five genes that have been analyzed in detail is remarkably varied (Table 2). Our screen initially identified two major classes of rhythmically expressed genes — those expressed at peak levels in the pre-dawn or early morning (Dreg-1 to Dreg-3), and a much larger set (Dreg-5 to Dreg-21) expressed at peak levels in the evening like per mRNA. Subsequent circadian characterization of the three morning genes revealed, however, that their oscillatory behavior was heterogeneous and complex, and that each was differentially dependent both on the endogenous,
per-dependent circadian oscillator and on external time cues of feeding time and light–dark cycle. Of the three morning genes, only Dreg-1 was strictly dependent on the per gene for its rhythmic expression. However, its phase of expression could be dissociated from that of per mRNA by altering the feeding time. In contrast, Dreg-3 mRNA was capable of robust oscillation in the absence of per gene function in flies maintained in a light–dark cycle, but was also capable of weak oscillation in the absence of external time cues in the presence of wild-type per gene function. Dreg-2 oscillation was the most complex observed, being dependent in a complex way on the presence of a lesser extent, on the light–dark cycle, on timed feeding and, to a lesser extent, on the function of the per gene. We have also investigated the oscillatory behavior of one of the evening genes identified in the screen (Dreg-3), whose mRNA is expressed precisely in phase with per mRNA [31]. Dreg-5 oscillation is dependent on per gene function, and its phase of expression could not be dissociated from that of per mRNA under altered environmental conditions (see Table 2). Dreg-5 protein, however, oscillates with a phase markedly different from that reported for Per protein [12].

The five cycling Drosophila genes that have been studied in detail therefore demonstrate five different rhythms and/or dependencies on internal and external oscillatory cues. Although the very low abundance and large size of

![Fig. 6.](image_url)

(a) Sequence of Dreg-3. The nucleotide sequence of Dreg-3 derived from clone pDREG3BS is shown along with the deduced amino-acid sequence of Dreg-3 protein. The initiation codon was selected as the first methionine codon in the long open reading frame. The region of homology to the FAD-binding domain of dihydroorotate dehydrogenase is shown in a grey box. The two cysteine-rich regions of homology to 4Fe–4S iron sulfur binding domains are underlined.

(b) Comparison of the Dreg-3 protein sequence to the sequences of dihydroorotate dehydrogenase (DHOD) in various organisms.
the other evening Dregs have hampered our efforts at similar characterization of these genes, preliminary experiments suggest that they too are diverse, but that a subset may behave as a more homogeneous group. Dreg-6 to Dreg-10 and Dreg-15 behaved like Dreg-5 in that they continued to cycle in constant darkness, and oscillated independently of feeding time cues. Preliminary experiments suggest that the cycling of some of these genes is dependent on per function (R.N.V.G. and H.B., unpublished observations). A subset of the evening Dregs, including Dreg-6, Dreg-7 and Dreg-9, also appeared to be very sensitive to caging conditions in a manner analogous to Dreg-1.

Our experiments establish that per influences the circadian expression of genes other than itself. We have also found, however, that only a subset of rhythmically expressed genes are tightly coupled to per. For each of the morning genes it appears that per functions in conjunction with extrinsic cues to determine the circadian expression patterns. In contrast, cycling of the evening gene Dreg-5 is tightly coupled to per [31]. It will be of interest to dissect the regulation of some of the Dreg genes in biochemical detail to determine whether there is a direct influence of Per on the expression of these genes, and how control by per and by various extrinsic cues are integrated to generate different patterns of rhythmic gene expression. It will also be important to further characterize the cycling profiles of the other evening genes, to learn how extrinsic cues and per influence their expression, and to obtain a refined estimate of the number of ‘clock-controlled genes’ whose cycling is tightly linked to per.

Roles of cycling gene expression

Why are certain genes expressed with diurnal rhythms? Some, like per, may be part of the internal time-keeping apparatus. For others, there is presumably some selective advantage to restricting expression to particular times of day. Many plant genes, including the chlorophyll-binding proteins, catalase and others, have been shown to undergo diurnal regulation of expression [19]; in these cases, the peak levels of expression are during the day when sunlight is available for photosynthesis. If a selective advantage is created by restricting expression of a gene to a particularly advantageous time, one would expect diurnally gated gene expression of particular genes to be conserved across phylogeny. In this regard, it is interesting that Dreg-1 was determined to be the alcohol dehydrogenase gene (Adh), as alcohol dehydrogenase activity is expressed with circadian rhythmicity in mammals [24,38]. This is the first gene identified with a circadian oscillation in expression that is conserved across phylogenetic classes. However, it should be noted that in the nocturnal mammals, where circadian variation of the enzyme activity has been described, alcohol dehydrogenase activity is maximal at the end of the subjective day and the beginning of the subjective night. In contrast, Dreg-1 (Adh) mRNA was found to be maximally expressed during the early day. Flies living in the wild are diurnally active, and feed on decaying fruit during the day. In both flies and mammals, it may be beneficial to trigger production of the detoxification enzyme prior to the actual feeding event.

As flies are active during the day, we were surprised to find that the vast majority (85%) of cycling transcripts were maximally expressed in the evening. Why so many of the cycling genes are maximally expressed when the flies are behaviorally inactive is unclear. It is also intriguing that almost all of the cycling genes we identified are large transcripts of low abundance (see Table 1).

Given that there is some advantage to restricting gene expression to particular times of the day, why do such varied and multiple mechanisms exist to achieve this end? For example, the per gene appears to be essential for the diurnal expression of Dreg-1 and Dreg-5, but is dispensable for diurnal Dreg-3 expression. A similar phenomenon is seen at the behavioral level. Diurnal rhythms of activity are observed both in wild-type flies kept in constant darkness and in per0 flies kept in a light–dark cycle [2,39]; two independent mechanisms clearly exist to generate diurnal rhythmicity of behavior in the fly. Although the present set of experiments do not answer this question, two hypotheses can be suggested. Firstly, although exogenous cues may be adequate to drive many aspects of circadian gene expression, certain genes may benefit from regulation within a window of time during the day that is effectively constant with respect to environmental conditions, or for which the environmental cues are not highly dependable. Such genes may rely more on the function of an endogenous circadian pacemaker than on exogenous cues. A second hypothesis is that certain genes may rely on both per function and external cues to modulate their expression in a circannual fashion. The overlapping influences of the circadian pacemaker and photic stimulation in modulating individual gene expression could therefore be the mechanism of photoperiodic induction of such circannual events as ovarian diapause in certain species of Drosophila [40]. Left unexplained by either hypothesis, however, are the genes like Dreg-2 that show rhythmic expression patterns that are dependent on complex interactions between per and the various environmental controls.

The functions of most of the Dreg genes are not apparent from their sequences. That 19 of the 20 oscillating cDNAs appear to represent new Drosophila genes suggests that these genetic functions, which may be either part of the clock or functions that are advantageously sequenced to particular times of day, have not been addressed at a molecular level. The biochemical roles of Dreg-2 and Dreg-3 are not revealed by their complete coding sequences as neither of these gene products shows substantial similarity to those of previously cloned genes, and only Dreg-3 protein has any clearly recognizable functional motifs. Dreg-3 contains two iron-sulfur-binding motifs and a potential FAD-binding region. Such domains are found in a number of reductases, including ferredoxin...
and cytochrome systems, as well as in the transferrin mRNA-binding protein cis-aconitase, suggesting that Dreg-3 may have a role in either metabolism or gene regulation [41].

The collection of Dreg genes will be valuable in further studies of the mechanisms of circadian rhythms in Drosophila. They can provide molecular genetic markers of events that lead from the central circadian pacemaker to the expression of behavior. Analysis of their oscillatory behavior in each of the different Drosophila mutants that affect circadian rhythms and behavior should allow ordering of the Dregs with respect to these genes and elucidation of the genetic pathway for circadian rhythms in Drosophila. Establishing the functions of the Dreg genes by mutational analysis and by characterization of their protein products will also be invaluable in determining the role of the circadian expression of these genes and their functions in generating circadian behavior and physiology.

Conclusions

Our large scale survey of the daily expression patterns of individual Drosophila genes identified 20 genes that show significant daily rhythms of gene expression. Circadian control of gene expression is therefore a significant but limited phenomenon in the fly head. Circadian gene expression is not a unitary phenomenon but rather is mediated by multiple mechanisms, which include the timing of the daily light–dark cycle, the timed availability of food and the function of the per gene and endogenous oscillator. Although a subset of rhythmically expressed genes is primarily or exclusively dependent on per gene function, many cycling genes appear to depend on all three types of input for their daily expression patterns. All but one of the 20 cDNAs appear to identify new genes. These cycling genes provide a valuable new molecular tool for elucidating the genetic pathway from per and the central time-keeping apparatus to the circadian behavior and physiology that it controls.

Materials and methods

Fly strains, maintenance and collection

D. melanogaster strains used in these experiments were either Canton-S, or y per01 derived from an outcross of y per01, rp506 to Canton-S. For the large-scale screen, 60 g (~6 x 10⁹) wild-type Canton-S flies were seeded at one week of age into each ~201 plexiglass population cage, and maintained in LD 12:12 with overhead 60 W tungsten incandescent lighting during light phase, and 15 W tungsten light with a red (Wratten 1A) filter during dark phase. Cages were kept at 25 °C and 55 % humidity in a double-door isolation room. Flies were fed baker’s yeast overlaid on apple-juice agar plates. Excess yeast was used such that some food remained on the plates for the full 24 h. Food trays were changed at ZT 17 under 15 W red-filtered light. Following five days of entrainment under LD 12:12 lighting, flies were collected by CO₂ anesthesia of the entire cage followed by quick-freezing in liquid nitrogen. Two cages of flies were collected for each timepoint.

For smaller scale experiments, 250–300 mg of flies were maintained at 24 °C and 55 % relative humidity in each 200 ml plastic bottle containing cornmeal–molasses agar medium overlaid with yeast. LD 12:12 was provided by either incandescent or fluorescent lighting alternating with complete darkness in light-tight incubators. Flies were transferred to fresh bottles three to four days prior to collection, and were collected serially from bottles kept in the same incubator. 10–15 bottles of each genotype were collected at each time point under 10 W red-filtered lighting. Flies were collected directly onto dry ice.

RNA purification and northern blotting

Frozen fly heads were separated from antennae and other body parts by vigorous vortexing and then purified by sieving over #25 and #40 US Standard brass sieves. Any remaining body parts were removed manually. Total RNA was prepared from 2 g fly heads per timepoint using chaotropic salt homogenization and LiCl precipitation [42]. Poly A⁺-containing RNA was purified by oligo(dT) cellulose chromatography [43]. RNA concentrations were determined by A₂₆₀ spectrometry.

For northern blots, 15 or 20 µg total RNA was loaded per lane on a 0.8 % agarose gel containing 2.2 M formaldehyde and run in MOPS buffer [43]. RNA was capillary blotted overnight to Nytran membrane and bound to the membrane by UV crosslinking at 1200 µJ per cm². Hybridization conditions were: 50 % formamide, 6x SSC, 5x Denhardt's reagent, 0.2 % SDS, denatured salmon sperm DNA at 100 µg ml⁻¹ and cRNA probe at 2 x 10⁶ cpm ml⁻¹. Hybridizations were carried out at 65 °C in a rolling-bottle incubator for at least 12 h. The final wash was in 0.1x SSC, 0.2 % SDS at 65 °C. Blots were exposed to Kodak XAR film with intensifying screen or quantified on a Molecular Dynamics Phosphorimager using ImageQuant v. 3.22 software.

Production of cRNA probes

PCR amplification of the cDNA insert in each phage stock from the head-not-embryo cDNA collection [28] was performed using ~3 x 10⁶ pfu phage and 30 pmol of the primers SWAJ T7 (5' TCG AAA TTA ATA CGA CTC ACT ATA GGG) and SWAJ SP6 (5' ACA CAT ACG ATT TAG GTG ACA CTA TAG). SWAJ T7 contains a bacteriophage T7 promoter and SWAJ SP6 contains an SP6 promoter. Standard PCR conditions [43] were used for 30 cycles of 1 min denaturation at 94 °C, 2 min annealing at 55 °C, and 3 min extension at 72 °C. Products were separated on a 1.5 % agarose gel containing 2.2 M formaldehyde and run in MOPS buffer [43]. RNA was capillary blotted overnight to Nytran membrane and bound to the membrane by UV crosslinking at 1200 µJ per cm². Hybridization conditions were: 50 % formamide, 6x SSC, 5x Denhardt's reagent, 0.2 % SDS, denatured salmon sperm DNA at 100 µg ml⁻¹ and cRNA probe at 2 x 10⁶ cpm ml⁻¹. Hybridizations were carried out at 65 °C in a rolling-bottle incubator for at least 12 h. The final wash was in 0.1x SSC, 0.2 % SDS at 65 °C. Blots were exposed to Kodak XAR film with intensifying screen or quantified on a Molecular Dynamics Phosphorimager using ImageQuant v. 3.22 software.

To generate probes for per mRNA, the 640 bp Eor1-HindIII fragment extending from the 5' end of the p49 transcript [45] was subcloned into pBluescript SK+ (Stratagene). The resulting clone, pRP49BSSK, was cleaved with Eor1 and transcribed in vitro with T7 RNA polymerase as above. To generate probes for per mRNA, the full-length cDNA of the 'A' form of per, pCDPerF+ [46], was digested with PstI and Eor1, and the resulting 0.6 kb fragment (base
Cloning of full-length cDNAs and DNA sequencing

PCR products from the original phage DNA clones of the Dreg genes were subcloned into the XhoI and EcoRI sites of pBluescript SK+, and sequenced by chain termination sequencing using T7 and T3 primers. Full-length clones of Dreg-2 were obtained by screening an adult fly head cDNA library in the lambda-EXLX vector [48] using the original cDNA clone as probe. The full-length cDNA was then in vivo subcloned into pEXLX yielding p2H12EXLX. An Apal–SacI fragment containing the entire cDNA was subcloned into pMOB to generate p2H12MOB. The insert was sequenced on both strands using Tn3 transposon-facilitated DNA sequencing [49].

A partial cDNA of Dreg-3 (pDREG3EXLX) was obtained from the same adult-head lambda EXLX cDNA library, using the Dreg-3 cDNA as probe. A full-length clone (pDREG3BS) was obtained from another adult-head cDNA library in the vector lambda-ZAP (provided by T. Schwarz, Stanford University) by high-density screening of the library using the partial cDNA as probe, followed by PCR from the 5’ end of the initial cDNA into the vector polylinker to identify full-length clones [50]. Both cDNAs were sequenced completely on both strands, using a combination of transposon-facilitated DNA sequencing and directed sequencing with custom oligonucleotide primers.

Database searches were performed using the IntelliGenetics Suite programs with the GeneBank and EMBL databases.

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